PROTEIN PHOSPHATASE INHIBITORS DISRUPT THE CYTOSKELETON AND BILE SALT TRANSPORT IN CLUSTERS OF ISOLATED POLARIZED SKATE (*RAJA ERINACEA*) HEPATOCYTES

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Microcystins are hepatotoxic heptapeptide compounds produced by aquatic cyanobacteria which potently inhibit protein phosphatases 1 and 2A (MacKintosh et al., *FEBS Letters* 264:187-192, 1990). We have recently demonstrated that treatment of skate hepatocytes with microcystin radically alters the cytokeratin and actin filament cytoskeletons (Henson et al., *Bulletin, MDIBL* 37:6-7, 1998), a result which has also been reported in mammalian hepatocytes (Toivola et al., *J. Cell Sci.* 110:23-33, 1997). In the present study we have extended this work by examining the affects of the ser/thr phosphatase inhibitors calyculin A and okadaic acid on the skate hepatocyte cytoskeleton. In addition we have investigated whether any of these phosphatase inhibitors affect the transport of bile salt in clusters of isolated polarized skate hepatocytes. These clusters provide an excellent model system due to their maintenance of structural and functional polarity *in vitro*.

Clusters of hepatocytes were isolated from skate liver and maintained in culture according to Smith et al. (*J. Exp. Zool.* 241:291-296, 1988). Hepatocytes were treated for at least 30 minutes with 1-5 μM microcystin-LR (or -YM), 0.5-1 μM calyculin A, or 0.5-1 μM okadaic acid in elasmobranch Ringer's at 15°C. Immunofluorescent localization of cytoskeletal proteins was carried out as described in Henson et al. (*J. Exp. Zool.* 271:273-284, 1995). Cells were stained for actin filaments with either rhodamine-phalloidin or a mouse monoclonal antibody against a conserved epitope of actin. For microtubule localization, cells were labeled with either a mouse monoclonal anti-tubulin or a rabbit polyclonal anti-tubulin. For cytokeratin localization, cells were stained with a mouse monoclonal antibody raised against trout cytokeratins (clone 2E11, a generous gift of Dr. Jon Holy, U. of Minnesota Medical School) or a mix of the AE1/AE3 antibodies against mammalian cytokeratins (Woodcock-Mitchell et al., *J. Cell Biol.* 95:580-588, 1982). Bile salt transport was studied by exposing clusters to Ringer's containing 1 μM of the fluorescent bile salt derivative NBD-taurocholate, according to the methods of Miller et al. (*Am. J. Physiol.* 270:G887-896, 1996). Fluorescently labeled clusters were viewed using a 40X (1.0 NA) water-immersion objective lens on an Olympus Fluoview laser scanning confocal microscope.

Okadaic acid and calyculin A both generated cytoskeletal restructuring similar to that seen previously with microcystin treatment. The tangle of cytokeratin filaments present in the deep cytoplasm of control cells collapsed around the canalicular region upon phosphatase inhibition. The pericanalicular actin cytoskeleton of control cells was transformed into a series of punctate foci indicative of canalicular contraction and/or fragmentation. It is interesting to note that the microtubule array seen in control cells - microtubules emanating from the apical membrane in meridional-like pattern - was not greatly altered by phosphatase inhibition by any of the agents. NBD-TC transport results were recorded as the average of the percent fluorescence-positive canalicular lumens of the total clusters counted (minimum of 50 clusters per condition), with at least five separate transport experiments being conducted for each treatment condition (Figure 1). The phosphatase inhibitors all greatly reduced the ability of the skate hepatocytes to secrete the NBD-TC into the canalicular lumens. However, the treated cells did display punctate fluorescent staining indicating that the cells retained the ability to take up the NBD-TC. In one series of

experiments, microcystin was added to clusters which had already been loaded with NBD-TC. The lumens of these clusters demonstrated a clear fragmentation, suggesting that the microcystin-induced changes seen in the pericanalicular cytoskeleton definitely affect lumenal architecture.

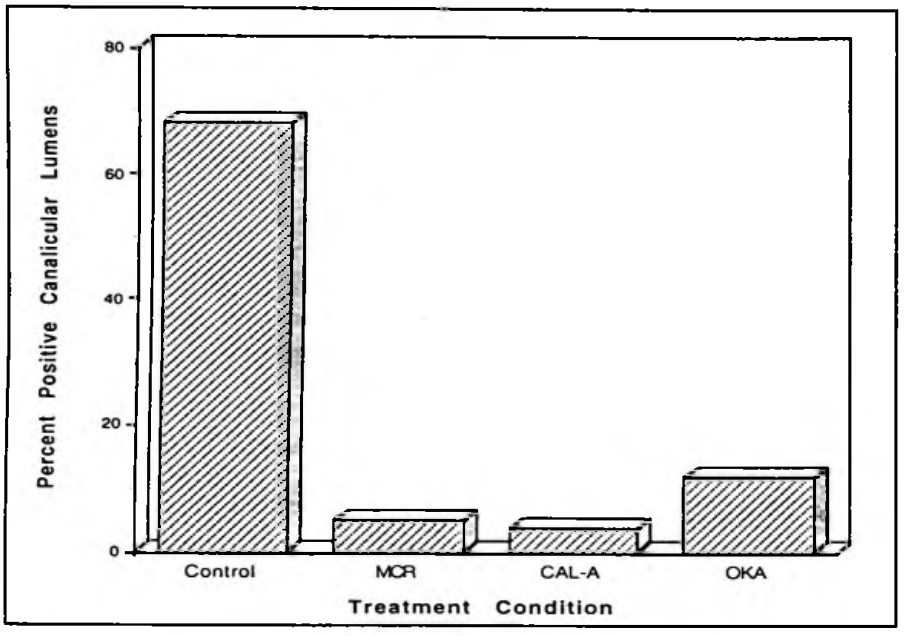

Figure 1: Results of NBD-TC transport experiments reported as an average of the percent of canalicular lumens which were positive for the presence of the fluorescent bile salt. Averages were based on at least five separate experiments. MCR = 5 μM microcystin; CAL-A = 1 μM calyculin; OKA = 1 μM okadaic acid

The results of our study indicate that ser/thr protein phosphorylation levels play a fundamental role in regulating the organization of the pericanalicular actin cytoskeleton, the arrangement of the cytokeratin filament array, and the process of bile salt transport within skate hepatocytes. These changes might be attributable to PP2A inhibition alone since okadaic acid largely inhibits PP2A while microcystin and calyculin A inhibit both PP1 and PP2A (we are currently performing the phosphatase analysis to make sure this is the case for these treatments in the skate cells). Given that bile salt transport has been shown to be microtubule dependent in skate hepatocytes (Henson et al., 1995, ibid), it is interestingly that the microtubule cytoskeleton appears to not be dramatically altered in response to phosphatase inhibition, a result which has also been reported in mammalian hepatocytes treated with microcystin (Hamm-Alvarez et al., Am. J. Physiol., 271:C929-C943). One possibility is that the inhibitors are interfering with microtubule-medicated vesicle movements (see Hamm-Alvarez, ibid) and/or the actual apical secretion process. Our transport results do suggest that the disruption of biliary excretion may be one of the main mechanisms underlying the potent hepatotoxicity of microcystin.

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